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## The Effects of a Nonimmunogenic Form of Murine Soluble Interferon- $\gamma$ Receptor on the Development of Autoimmune Diabetes in the NOD Mouse

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### ABSTRACT

Previous studies have shown that *in vivo* treatment with anti-interferon- $\gamma$  (anti-IFN $\gamma$ ) monoclonal antibodies (mAbs) prevents the development of autoimmune diabetes in NOD mice. Although these findings anticipate that specific anti-IFN $\gamma$  therapies may be useful for the prevention/treatment of human insulin-dependent diabetes mellitus, there are several reasons why the use of anti-IFN $\gamma$  mAb may be difficult in the clinical setting. With the aim to develop alternative forms of specific anti-IFN $\gamma$  therapies, we recently produced a non-immunogenic form of the soluble IFN $\gamma$  receptor (sIFN $\gamma$ R) that binds and neutralizes murine IFN $\gamma$  with an affinity higher than that of anti-IFN $\gamma$  mAb. In this study we compared the efficacy of sIFN $\gamma$ R to that of two anti-IFN $\gamma$  mAbs (XMG 1.2 and AN-18) in the prevention of spontaneous and accelerated (cyclophosphamide-induced) forms of autoimmune diabetes in NOD mice. The results show that in the

spontaneous model, sIFN $\gamma$ R could prevent histological and clinical signs of autoimmune diabetes as efficiently as the two mAbs. Under *ex vivo* conditions, sIFN $\gamma$ R exhibited a more powerful modulatory effect than XMG1.2 mAb on cytokine secretion from splenic lymphoid cells, which resulted in a significant reduction of Concanavalin A-induced IL-2 secretion and an augmented release of both unstimulated and lipopolysaccharide-induced IL-6. Moreover, although both mAbs were immunogenic and elicited formation of high titers of anti-rat IgG, sIFN $\gamma$ R did not induce antibody formation. Unexpectedly, in the cyclophosphamide-induced model, sIFN $\gamma$ R turned out to be less effective than either of the two anti-IFN $\gamma$  mAbs. Taken together, these data support the role of IFN $\gamma$  in the pathogenesis of NOD mice, but, more importantly, suggest that a nonimmunogenic approach is possible to the diminution of the effects of IFN $\gamma$  in this model. (*Endocrinology* 137: 5587–5575, 1996)

**I**NTERFERON- $\gamma$  (IFN $\gamma$ ) is a cytokine produced by T lymphocytes and natural killer cells that exhibits pleiotropic effects on the immune system (see Ref. 1 for review). Several lines of evidence suggest that IFN $\gamma$  may favor both systemic and organ-specific autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, multiple sclerosis, autoimmune thyroid diseases, and type 1 diabetes (insulin-dependent diabetes mellitus (IDDM), autoimmune diabetes) (see Refs. 1–3 for reviews). In particular, the observation that anti-IFN $\gamma$  monoclonal antibodies (mAbs) prevent the development of hyperglycemia in animal models of IDDM such as the NOD mouse (4–6), the BB rat (7), and the mouse made diabetic with multiple doses of streptozotocin (8) anticipates that blockade of endogenous IFN $\gamma$  with specific antagonists may also exert beneficial effects in human IDDM.

To date, in experimental studies conducted in animal models of immunoinflammatory diseases, IFN $\gamma$  bioactivity has primarily been inhibited by treatment with mAbs directed against the cytokine or its receptor. However, specific anti-IFN $\gamma$  therapy with mAbs may not be easily feasible in the clinical setting. As observed in transplantation studies with OKT3 mAb, the heterologous origin of the protein often

elicits a humoral immune response consisting of antixenotypic and antiidiotypic antibodies capable of reducing the bioactivity of the mAb (9, 10). Attempts to diminish the immunogenicity of mAbs in humans by creating chimeric (mouse-human) or fully humanized mAbs have in some cases been unsuccessful, as these mAbs may still induce the production of both neutralizing and antiidiotypic antibodies (11, 12). Theoretically, the prolonged treatment with mAb can also lead to immune complex formation, the deposition of which may further enhance local and systemic immunoinflammatory reactions through activation of the complement pathway.

As IFN $\gamma$  acts through binding to specific receptors (IFN $\gamma$ R) expressed on the surface of the target cells, an alternative form of specific anti-IFN $\gamma$  therapy may consist of administering a soluble form of the IFN $\gamma$ R. This would neutralize the endogenous IFN $\gamma$ , thus abrogating its immunomodulatory effects. For this purpose, we recently produced and characterized a soluble form of the mouse IFN $\gamma$ R (sIFN $\gamma$ R) that is not immunogenic, is capable of neutralizing murine IFN $\gamma$  bioactivity *in vitro* and *in vivo*, has an affinity higher than that of anti-IFN $\gamma$  mAbs (13, 14), and prevents SLE-like syndrome in the (NZB $\times$ NZW)F1 mouse (15). Importantly, even during prolonged (14 days) administration, sIFN $\gamma$ R does not induce antibody formation in BALB/c mice with doses up to 100  $\mu$ g/day (14).

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In this study we evaluated the effects of prolonged prophylactic treatment with sIFN $\gamma$ R on the development of spontaneous and accelerated [cyclophosphamide (CY)-induced] forms of autoimmune diabetes in NOD mice, and the results were compared with those obtained using either of the two rat antimouse IFN $\gamma$  mAbs, XMG1.2 and AN-18. The data demonstrate that prophylaxis with sIFN $\gamma$ R is as effective as either of the two anti-IFN $\gamma$  mAbs in preventing spontaneous, but not CY-induced, diabetogenesis in NOD mice. Moreover, although both XMG1.2 and AN-18 provoked the formation of antirat IgG upon prolonged (8 weeks) treatment, no significant rise in the serum titer of anti-sIFN $\gamma$ R antibody was observable in NOD mice treated for the same time with sIFN $\gamma$ R.

## Materials and Methods

## Mice

Female NOD/Lt mice were provided by Jackson Laboratories (Bar Harbor ME). The mice were kept under standard laboratory conditions (nonspecific pathogen free) with free access to food and water and were cared for according to the guidelines of the local committee for animal research. They were allowed to adapt for at least 1 week to their environment before commencing the experiment. Mice were defined as diabetic on the basis of 2 consecutive days of glycosuria (Tes-tape, Eli Lilly Co., Indianapolis, IN), followed by fasting glycemia above 210 mg/dl. Under these experimental conditions, 60–75% of female NOD/Lt mice develop diabetes between 12–35 weeks of age.

Anti-IFN $\gamma$  mAbs

The XMG1.2 mAb is a rat IgG1 anti-mouse IFN $\gamma$  (16); its neutralizing titer was 10 U mouse IFN $\gamma$ /4 ng protein as assessed on L929 cells. The rat mAb AN-18 is an IgG2a anti-mouse IFN $\gamma$  previously produced and characterized by Prat and colleagues (17). The antibody neutralizes the antiviral activity of both natural and recombinant mouse IFN $\gamma$ , but does not react with murine IFN $\alpha$  or  $\beta$ . The neutralizing titer of the AN-18 mAb was 10 U mouse IFN $\gamma$ /12 ng protein, as assessed using L929 cells.

Murine sIFN $\gamma$ R

The extracellular domain of the mouse IFN $\gamma$ R (sIFN $\gamma$ R) was expressed in Sf9 insect cells infected with recombinant baculoviruses. The R was expressed, purified, and biochemically characterized, as described previously (14, 18).

## Protein and chemicals

Control rat IgG, Concanavalin A (Con A), and lipopolysaccharide (LPS) were obtained from Sigma Chemical Co. (St. Louis, MO). CY was provided by Schering-Plough (Milan, Italy).

## Experimental design

**Spontaneous IDDM.** Euglycemic female NOD, randomly allocated into different groups, received either anti-IFN $\gamma$  mAbs or sIFN $\gamma$ R starting at the 4th or the 16th week of age. Because insulinitis is absent in 4-week-old NOD mice and is actively ongoing in virtually all animals by the age of 16 weeks (19), this experimental approach allowed us to investigate the role of endogenous IFN $\gamma$  in both afferent and efferent phases of the diabetogenic process in the NOD mouse.

For the early prophylactic treatment, four groups of 4-week-old female euglycemic NOD mice were created that were treated with PBS (group A), irrelevant rat IgG (group B), XMG1.2 (group C), AN-18 (D), or sIFN $\gamma$ R (group E) according to the doses and treatment schedule described in Fig. 1. Irrelevant rat IgG were used as controls for either XMG1.2 or AN-18 mAbs, and PBS was used as the control for sIFN $\gamma$ R. Although PBS has been previously employed by ourselves and others as a control for soluble cytokine receptors when these reagents were used in various models of autoimmune diseases (20–22), it is worth

mentioning that PBS does not provide a suitable control for the soluble receptor, which should, instead, consist of fragments of the receptor that do not bind the cytokine *in vitro* or *in vivo*. Because these fragments are not yet available for sIFN $\gamma$ R, we decided to use PBS as control for sIFN $\gamma$ R.

After 9 weeks of continuous treatment, the mice were killed, and their pancreata specimens were collected and examined for the prevalence and severity of insulinitis. Mice becoming diabetic before this age were killed and not included in the histological examination.

Moreover, to evaluate the effects of these IFN $\gamma$  inhibitors on the cytokine secretory capacity of NOD mice, spleens were collected at death from individual mice from groups B, C, and E, passed through a sterile sieve, and suspended in Hanks' Balanced Salt Solution (HBSS). Splenic lymphoid cells (SLC), obtained and cultured for 48 h at 37°C with 5% CO<sub>2</sub>, as described previously (23), were either unstimulated or stimulated with Con A or LPS. At the end of the culture period, the cells were centrifuged, and the supernatant collected was aliquoted at –20°C until assayed for the content of interleukin-2 (IL-2), IL-4, IL-6, IL-10, IFN $\gamma$ , and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ). These cytokines were measured using specific mouse-specific solid phase enzyme-linked immunosorbent assay (ELISA), purchased by Biosource (Camarillo, CA), for IL-2, IL-4, IL-6, IL-10, and IFN $\gamma$  and Endogen (Boston, MA) for TNF $\alpha$ . Undiluted or 2-fold (IL-6) diluted supernatants were run in duplicate and assayed following the manufacturers' instructions. The limits of sensitivity of the assays were 13 pg/ml for IL-2, 5 pg/ml for IL-4, 8 pg/ml for IL-6, 13 pg/ml for IL-10, 1 pg/ml for IFN $\gamma$ , and 15 pg/ml for TNF $\alpha$ .

For the late prophylactic treatment, 16-week-old euglycemic female NOD mice were divided into 5 groups (F, G, H, I, and J) and treated with PBS (F), irrelevant rat IgG (G), XMG1.2 (H), AN-18 (I), or sIFN $\gamma$ R (J) according to the experimental protocol shown in Table 1. Mice were screened for diabetes once a week. The mice from groups F, G, and H were killed either at the onset of diabetes or at the end of the experiment (24 weeks of age), and their pancreata specimens were collected for histological analysis. Diabetic mice from groups I and J were also killed at the onset of the disease; in contrast, only 4 of 9 and 7 of 17 of the remaining euglycemic animals from the sIFN $\gamma$ R- and AN-18-treated groups were killed at the end of the study for histological examination of the  $\beta$ -cells, and their SLC were used for the IDDM transfer experiment (see after). After treatment withdrawal, the remaining euglycemic mice were kept for a 3-month follow-up period to determine whether sIFN $\gamma$ R or AN-18 prophylaxis afforded temporary or permanent protection from the disease.

**CY-induced IDDM.** Another set of experiments was performed to evaluate the effects of sIFN $\gamma$ R on the accelerated model of diabetes that can be provoked in NOD mice with CY. For this purpose, seven groups of 100- to 120-day-old female NOD mice were studied; the animals were injected with CY and treated according to the experimental design shown in Table 2. Mice were screened for the development of diabetes on days 14 and 15 after CY challenge. On day 15, all mice from these groups were killed, and their pancreata specimens were analyzed to evaluate the extent and severity of the insulinitis process.

**IDDM transfer studies.** Finally, we wondered whether prolonged prophylactic treatment of spontaneously diabetes-prone NOD mice with sIFN $\gamma$ R or anti-IFN $\gamma$  AN-18 mAb prevented IDDM through generation of suppressor cells. To prove this, we used the accelerated form of diabetes inducible in newborn NOD mice by transferring splenic lymphoid cells from acutely diabetic NOD mice (24). In this study, newborn NOD mice were preinjected iv with splenic lymphoid cells ( $3 \times 10^7$ ) from euglycemic sIFN $\gamma$ -treated NOD mice 12 h before the iv injection of spleen cells ( $3 \times 10^7$ ) from acutely diabetic female NOD mice. Two control groups were considered. One consisted of newborn NOD mice preinjected under the same experimental conditions with splenic lymphoid cells from 19-week-old euglycemic, PBS-treated, female NOD mice before transfer of spleen cells from acutely diabetic mice; in the other control group, newborn mice received only splenic lymphoid cells from acutely diabetic female NOD mice.

Measurement of humoral immune response to sIFN $\gamma$ R

The occurrence of anti-sIFN $\gamma$ R antibody was evaluated by solid phase ELISA as described previously (25). Microtiter plates were coated with sIFN $\gamma$ R in PBS. Different dilutions of serum samples from individual

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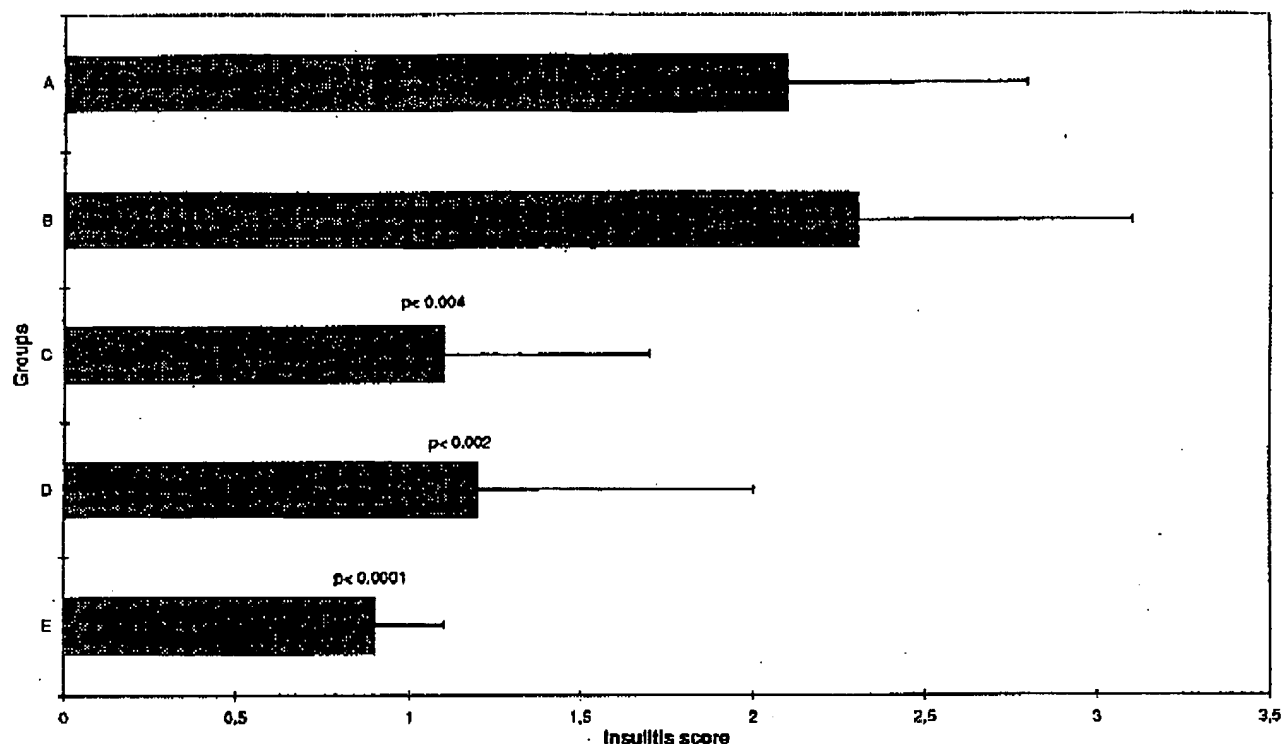


FIG. 1. Protection from insulinitis development in NOD mice by early prophylactic treatment with XMG 1.2 and AN-18 anti-IFN $\gamma$  mAbs or sIFN $\gamma$ R. Euglycaemic 4-week-old female NOD received for 9 consecutive weeks mouse anti-IFN $\gamma$  mAbs, XMG1.2 (group C; 100  $\mu$ g, twice a week, ip) or AN-18 (group D; 250  $\mu$ g, twice a week, ip), or sIFN $\gamma$ R (group E; 250  $\mu$ g/day, six times a week, ip). Control mice were treated with either PBS (group A 200  $\mu$ l, 6 times a week, ip) or irrelevant rat IgG (group B; 260  $\mu$ g, twice a week, ip). At 18 weeks of age, the mice were killed, and pancreata specimens were collected for histological analysis. For statistical analysis, group E was compared to group A, and groups C and D were compared to group A. Each group contained eight mice. Data are the mean  $\pm$  SD.

mice treated with either sIFN $\gamma$ R or PBS were incubated for 2 h at room temperature. After extensive washing, a 1:1500 dilution of an alkaline-phosphatase-conjugated sheep antimouse Ig was used as the second step reagent.

#### Measurement of the immune responses to XMG1.2 and AN-18 mAbs

The formation of murine antibodies to XMG 1.2 and AN-18 was studied by a solid phase ELISA previously described by Williams *et al.* (26). In brief, microtiter plates, coated for 1 h at room temperature with 5  $\mu$ g/ml of either XMG 1.2 or AN-18, were blocked and then incubated with serially diluted sera. After washing, horseradish peroxidase-conjugated goat antimouse IgG was used as the second antibody at a 1:3000 dilution in 2% bovine gamma globulin (BGG) and 1% BSA (75  $\mu$ l/well) and kept at room temperature for 1 h. After washing, the colorimetric reaction was developed by adding  $\alpha$ -phenylenediamine in PBS for 10 min. Readings were performed at 490 nm (Therrek, Flow Labs, Rockville, MD) after stopping the reaction with 2 N H<sub>2</sub>SO<sub>4</sub>. Results are expressed as mean OD values.

#### Measurement of unbound XMG1.2 mAb

Microtiter plates were coated with recombinant murine IFN $\gamma$  (10  $\mu$ g/ml), blocked, and then incubated with test sera. Goat antirat IgG-alkaline phosphatase conjugate was added, followed by substrate addition. Quantitation was obtained by reference to a sample of a known concentration of XMG 1.2. Results are expressed (micrograms per ml) as the mean ( $\pm$  SD) unbound XMG 1.2.

#### Histological examination of pancreatic islets

Histological examination of pancreatic islets was performed in a blind fashion by 2 pathologists unaware of the status and/or the treatment of the animals, as described previously (27). At least 10 islets were counted for each pancreas. The degree of mononuclear cell infiltration was graded as follows: 0, no infiltrate; 1, periductular infiltrate; 2, perislet infiltrate; 3, intraslet infiltrate; and 4, intraslet infiltrate associated with  $\beta$ -cell destruction. The mean score for each pancreas was calculated by dividing the total score by the numbers of islets examined.

#### Statistics

Results are expressed as the mean  $\pm$  SD. Data were analyzed using ANOVA. In situations considered significant, as appropriate, the *post-hoc* least significance difference was used to determine where pairwise differences existed. The incidence of diabetes among different groups was tested for significance using the  $\chi^2$  test with Yates' correction.  $P < 0.05$  was considered significant.

#### Results

##### Spontaneous IDDM

Upon early prophylactic treatment, sIFN $\gamma$ R prevents insulinitis development in NOD mice even more efficiently than XMG 1.2 anti-IFN $\gamma$  mAb, and it exerts more powerful immunomodulatory effects on cytokine secretion than XMG 1.2 mAb.

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EFFECTS OF sIFN $\gamma$ R ON DEVELOPMENT OF IDDMEndo • 1995  
Vol 137 • No 12**TABLE 1.** Experimental design and effects of prophylactic treatment with PBS, rat IgG, AN-18 or XMG 1.2 anti-IFN $\gamma$  mAb, and m sIFN $\gamma$ R on the development of spontaneous diabetes and insulinitis in NOD mice

Groups	No. of subjects	Treatment ( $\mu$ g)	Diabetes incidence (%)	Insulinitis scores
F	20	PBS	14/20 (70)	2.9 $\pm$ 0.8
G	18	Rat IgG (200)	13/18 (72.2)	3.1 $\pm$ 0.7
H	21	XMG 1.2 (200)	3/21 (14.3) <sup>a</sup>	2.8 $\pm$ 0.9
I	21	AN-18 (500)	4/21 (19) <sup>b</sup>	2.7 $\pm$ 0.8
J	11	m sIFN $\gamma$ R (100)	2/11 (18.2) <sup>c</sup>	2.9 $\pm$ 0.6

Sixteen-week-old euglycemic female NOD mice were treated for 8 consecutive weeks with two injections per week of rat IgG, AN-18, or XMG 1.2 or with three injections of PBS or sIFN $\gamma$ R. All treatments were given ip. For statistical analysis, groups H and I are compared to group F, and group J is compared to group F. Insulinitis scores are shown as the mean  $\pm$  SD. Significance was determined by  $\chi^2$  test with Yates' correction.

<sup>a</sup>  $P < 0.0008$  vs. group G.

<sup>b</sup>  $P < 0.002$  vs. group G.

<sup>c</sup>  $P < 0.02$  vs. group F.

As expected, clear signs of early insulinitis with peri-intra-islet infiltration of the  $\beta$ -cells were observed at the age of 13 weeks in most control NOD mice treated with either PBS or irrelevant rat IgG (Fig. 1). In contrast, although the incidence of insulinitis in the mice treated with XMG 1.2, AN-18, or sIFN $\gamma$ R was not different from that observed in control mice (data not shown), the process was significantly milder in the mice that received either of the two anti-IFN $\gamma$  mAbs and to an even greater extent in those treated with sIFN $\gamma$ R (Fig. 1).

Along with the development of spontaneous diabetes, NOD mice also exhibit an altered pattern of cytokine production *in vitro*, which might be implicated in the pathogenesis of the disease, with augmented secretion of IFN $\gamma$  and decreased release of IL-2, IL-4, and TNF- $\alpha$  (see Ref. 28 for a review). Thus, we wondered whether the antidiabetogenic action of the IFN $\gamma$  inhibitors could be related to a change in the cytokine secretory capacity of NOD mice.

As previously observed for the histological analysis, sIFN $\gamma$ R exhibited a more powerful modulatory effect than XMG1.2 mAb on cytokine secretion, which resulted in a significant reduction of Con A-induced IL-2 secretion and an augmented release of both unstimulated and LPS-induced IL-6. Hence, when Con A-induced, T cell-derived cytokines were considered, the only significant effect we noticed was the marked reduction of IL-2 secretion in the mice treated with sIFN $\gamma$ R compared to the PBS-treated control group (Fig. 2). A trend toward decreased release of IL-2 was also observed in the mice treated with XMG 1.2 mAb, but the effect was not statistically significant (Fig. 2). No significant change was observed among the three groups considered in the Con A-induced secretion of IFN $\gamma$  and IL-6 (Figs. 2 and 3). Con A-induced IL-4 and IL-10 secretion was always below the limit of sensitivity of the assays. In the same manner, unstimulated supernatants contained no detectable amounts of IL-2, IL-4, IL-10, and IFN $\gamma$ .

On the other hand, when LPS-induced, and thus primarily macrophage- and B lymphocyte-derived, cytokines were measured, we found that SLC obtained from both XMG 1.2- and sIFN $\gamma$ R-treated groups secreted significantly larger amounts of IL-6 than those obtained from the PBS-treated

**TABLE 2.** Experimental design and effects of the treatment with PBS, rat IgG, AN-18 or XMG 1.2 anti-IFN $\gamma$  mAb, and m sIFN $\gamma$ R on the development of CY-induced diabetes and insulinitis in NOD mice

Groups	No. of subjects	Treatment (mg)	Diabetes incidence (%)	Insulinitis scores
K	19	PBS	13/19 (68.4)	3.2 $\pm$ 0.6
L	19	Rat IgG (1)	6/19 (31.6)	3.1 $\pm$ 0.6
M	18	XMG 1.2 (1)	1/18 (5.6) <sup>a</sup>	2.8 $\pm$ 0.9
N	13	AN-18 (0.5)	5/13 (38.4)	2.9 $\pm$ 0.8
O	20	AN-18 (1.5)	2/21 (9.5) <sup>b</sup>	2.7 $\pm$ 0.9
P	13	m sIFN $\gamma$ R (0.25)	4/13 (30.7)	2.7 $\pm$ 0.8
Q	9	m sIFN $\gamma$ R (0.4)	5/9 (55.6)	2.6 $\pm$ 0.8

One hundred to 120 old euglycemic female NOD mice were challenged with CY (300 mg/kg) on day 0. Treatment was given daily from days -2 to 13 in groups K and P and on alternate days in group Q; the mice from the other groups were treated on days -2, 3, 6, and 9. All injections were given ip. For statistical analysis, groups P and Q are compared to group K, and groups M, N, and O are compared to group L. Insulinitis scores are shown as the mean  $\pm$  SD. Significance was determined by chi-square with Yates' correction.

<sup>a</sup>  $P < 0.03$ .

<sup>b</sup>  $P < 0.04$ .

control group. Here again, the effect was more evident for sIFN $\gamma$ R, which, unlike XMG1.2, could significantly increase the spontaneous release of IL-6 compared to that in PBS-treated controls (Fig. 3). No effects of either XMG 1.2 or sIFN $\gamma$ R on the Con A- or LPS-induced secretion of IL-10 and TNF- $\alpha$  could be determined, because the contents of these cytokines in cell supernatants were in each case below the limit of sensitivity of the assay we used. This was confirmed using two different ELISA kits for each cytokine (Biosource and Endogen; data not shown).

**Late prophylactic treatment with sIFN $\gamma$ R is as effective as XMG 1.2 anti-IFN $\gamma$  mAbs in preventing IDDM development in NOD mice.** As expected, IDDM developed in the majority of the NOD mice that had been treated with either PBS or irrelevant rat IgG for 8 consecutive weeks and served as control groups. In contrast, the incidence of the disease was substantially reduced in the three groups of mice treated with sIFN $\gamma$ R, XMG 1.2, or AN-18 (Table 1). However, at least for sIFN $\gamma$ R and AN-18, continuous administration seemed to be necessary for the antidiabetogenic effect to be maintained. Thus, animals from these 2 groups developed IDDM with similar incidence and comparable kinetics after treatment withdrawal, with 3 of 5 and 5 of 10 of the mice that had been treated with sIFN $\gamma$ R or AN-18, respectively, becoming hyperglycemic between the 5th and 10th weeks of the follow-up period.

A severe insulinitis process also occurred in the two control groups of NOD mice regardless of their clinical status (Table 1 and data not shown). In contrast to the high efficiency with which each of the three IFN $\gamma$  inhibitors blocked the clinical outcome of diabetes, neither of them offered clear protection from the development of insulinitis. So, although NOD mice treated with XMG 1.2, or AN-18 exhibited a trend toward less severe insulinitis compared to both PBS- and rat IgG-treated control mice, the difference was not statistically significant (Table 1).

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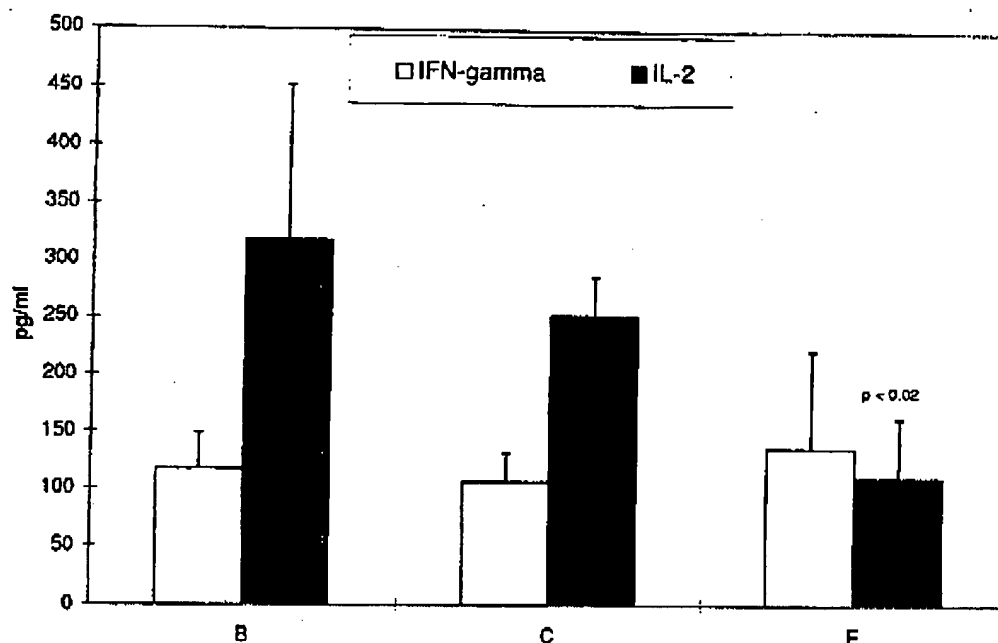


FIG. 2. *Ex vivo* effects of XMG 1.2 anti-IFN $\gamma$  mAbs and sIFN $\gamma$ R on Con A-induced secretion of IL-2 and IFN $\gamma$ . After 9 weeks of continuous treatment with irrelevant rat IgG (group B), XMG 1.2 anti-IFN $\gamma$  mAb (group C), or sIFN $\gamma$ R (group E), euglycemic NOD mice were killed, and individual spleens were collected. Splenic lymphoid cells were cultured for 48 h at 37°C in 5% CO $_2$  in the presence of Con A (4  $\mu$ g). The cytokine content of each culture supernatant was determined by ELISA. For statistical analysis, each group was compared to group B. Data are shown as the mean  $\pm$  SD.

## CY-induced IDDM

Both XMG 1.2 and AN-18 anti-IFN $\gamma$  mAbs, but not sIFN $\gamma$ R, suppress CY-induced diabetes in NOD mice. An accelerated form of autoimmune diabetes may be provoked in NOD mice by injecting them with one or two large doses (200–350 mg/kg daily) of CY at 100–120 days of age. CY seems to act by inhibiting suppressor cell function (29–31), thus allowing autoreactive cells to cause complete  $\beta$ -cell destruction and diabetes within 14–28 days after the first challenge. IFN $\gamma$  plays a central pathogenic role in this model (4, 5), and its release seems to occur in a more vigorous and abundant fashion than in the spontaneous form, as judged by *ex vivo* studies in which  $\beta$ -cells infiltrating lymphocytes secrete IFN $\gamma$  shortly after CY challenge (4). The requirement for much larger doses of anti-IFN $\gamma$  mAbs necessary for the prevention of CY-induced diabetes compared to spontaneous diabetes (4–6) (see below) further substantiates this experimental observation.

In our study, the majority of control NOD mice treated with either PBS or rat IgG have developed an acute form of IDDM with glycosuria, hyperglycemia, and severe insulinitis within 15 days after challenge with CY (Table 2). In contrast, confirming previous results (4, 5), treatment with anti-IFN $\gamma$  mAb XMG 1.2 or AN-18 significantly suppressed the development of diabetes (Table 2). The dose dependency of the phenomenon for AN-18 was investigated, and the results showed that its preventive action is clearly dose dependent, with the effect achieving statistical significance at the highest doses.

Unexpectedly, in contrast to the results obtained in the spontaneous model, the antidiabetogenic action of sIFN $\gamma$ R in the CY-induced form of diabetes was weaker than that afforded by either XMG 1.2 or AN-18 anti-IFN $\gamma$  mAbs. Thus, although a trend toward reduced incidence of diabetes *vs.* PBS-treated controls was noticed in the two groups of mice treated with sIFN $\gamma$ R, with the effect being more evident when sIFN $\gamma$ R was administered daily at the dose of 0.25 mg/mouse from 2 days before until 13 days after CY challenge (Table 2), the phenomenon was not statistically significant, possibly because of the smaller size of the experimental groups compared to that of the control group (Table 2).

Finally, neither the anti-IFN $\gamma$  mAbs nor sIFN $\gamma$ R diminished the insulinitis process in CY-challenged NOD mice, whose insulinitis score was only slightly, not significantly, milder than that in PBS-treated controls (Table 2). No significant difference could be found in the insulinitis score between CY-challenged NOD mice that did or did not develop IDDM (data not shown).

**IDDM transfer studies: blockade of endogenous IFN $\gamma$  with specific inhibitors does not protect NOD mice from diabetes development through generation of suppressor cells.** By 45 days after the injection of spleen cells from acutely diabetic NOD mice, an acute form of IDDM occurred in six of six newborn NOD mice; a trend toward a reduced incidence of the disease was observed in the group of mice preinjected with spleen cells from sIFN $\gamma$ R-treated NOD mice, only three of five of which became diabetic. However, the effect did not depend on the

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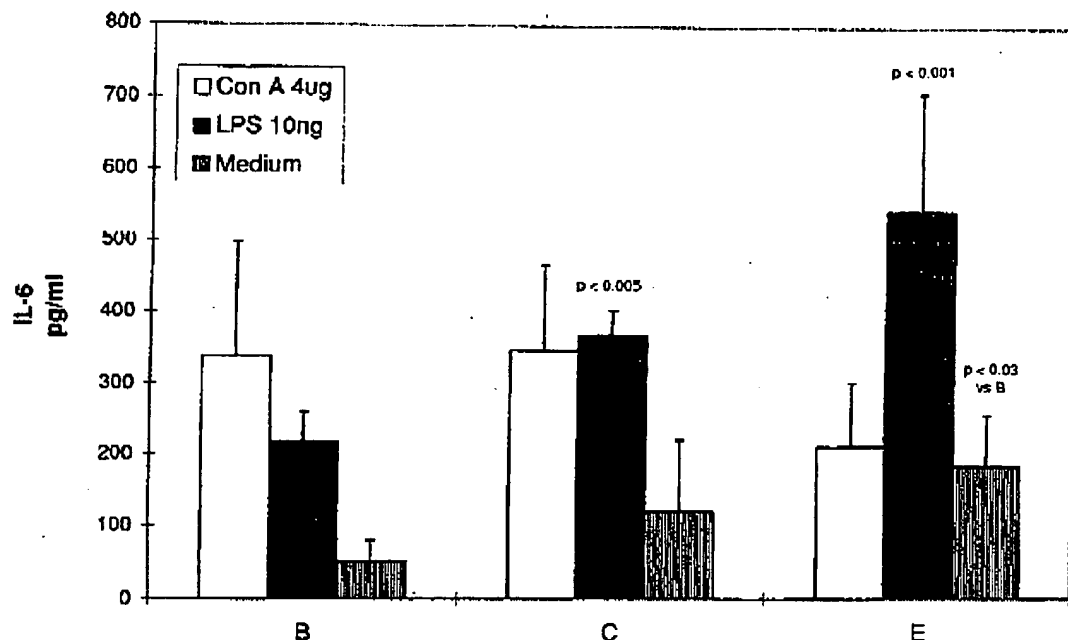
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FIG. 3. *Ex vivo* effects of XMG 1.2 anti-IFN $\gamma$  mAbs and sIFN $\gamma$ R on spontaneous, LPS-induced, and Con A-induced secretion of IL-6. After 9 weeks of continuous treatment with irrelevant rat IgG (group B), XMG1.2 anti-IFN $\gamma$  mAb (group C) or sIFN $\gamma$ R (group E), ouglycemic NOD mice were killed and individual spleens collected. Splenic lymphoid cells were cultured for 48 h at 37 C in 5% CO<sub>2</sub> in the presence or absence of LPS (10 ng) or Con A (4  $\mu$ g). IL-6 was measured by ELISA. For statistical analysis, each group was compared to group B. Data are shown as the mean  $\pm$  SD.

treatment received *in vivo*, as a reduced incidence of diabetes had also occurred in newborn NOD mice preinjected with spleen cells from PBS-treated NOD mice (four of seven).

**Immunogenicity of sIFN $\gamma$ R, XMG 1.2, and AN-18: upon prolonged treatment, anti-IFN $\gamma$  mAbs, but not sIFN $\gamma$ R, induce antibody production in NOD mice.** As shown in Fig. 4, a significant increase in the blood titers of murine IgG directed against XMG 1.2 and AN-18 was noticed in the NOD mice that received these mAbs. This increase first became appreciable after 3 weeks of treatment and was maximal at 6 and 7 weeks when it reached a plateau that was maintained until the end of the treatment. Interestingly, measurement of the levels of unbound XMG1.2 in the circulation of the NOD mice treated with this mAb revealed a progressive decline of unbound mAb that well paralleled the development of anti-rat IgG (see Fig. 5).

In contrast, NOD mice treated with sIFN $\gamma$ R showed only a slight elevation of specific Ig antibody that, even though more pronounced at 5 and 6 weeks, was not statistically significant (Fig. 4).

### Discussion

We demonstrated for the first time the efficacy of prolonged prophylactic treatment with sIFN $\gamma$ R in the prevention of histological and clinical signs of autoimmune diabetogenesis in NOD mice. Clinical, pathogenic, and immunopharmacological considerations worthy of attention may be drawn from our study.

The lack of immunogenicity of sIFN $\gamma$ R may have great

relevance for the potential use of this IFN $\gamma$  inhibitor in the management of human IDDM and possibly other autoimmune conditions. The biological relevance of antixenotypic antibody formation and its interference with anticytokine antibody therapy is emphasized by a recent study in which a rat antimouse IL-6 mAb is only effective in preventing SLE-like syndrome in (NZB $\times$ NZW)F1 mice if formation of antirat IgG by the mice is suppressed with a short course of tolerizing anti-CD4 mAb (32). In our study, although murine IgG directed against the two mAbs were produced from the mice treated for 8 weeks with either XMG 1.2 or AN-18, both of these mAbs protected NOD mice from diabetes development with a degree of protection comparable to that observed with the nonimmunogenic sIFN $\gamma$ R. This is probably due to the fact that a substantial increase in murine IgG directed against the two mAbs was first detectable in NOD mice 3 weeks after the first injection, a period within which endogenous IFN $\gamma$  may have been sufficiently antagonized by the mAbs so as to afford temporary blockade of disease progression and delay its onset. On the other hand, not even by sIFN $\gamma$ R treatment would complete and long lasting neutralization of endogenous IFN $\gamma$  be accomplished, because although it is not immunogenic, the short half-life it possesses (1 h after ip injection) (14) rapidly reduces its circulating levels and bioavailability.

Unexpectedly, when used in the CY-induced form of diabetes, sIFN $\gamma$ R offered only a slight and not significant preventive action, which contrasts with the clear-cut efficacy of anti-IFN $\gamma$  mAbs, previously demonstrated by two indepen-

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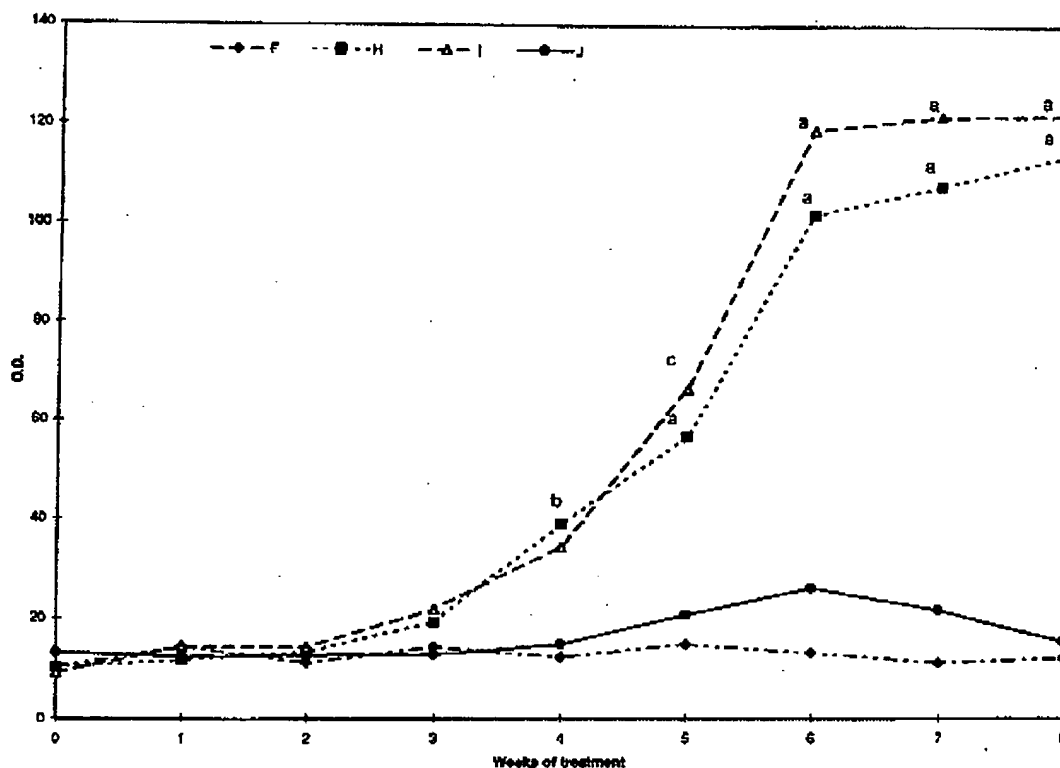
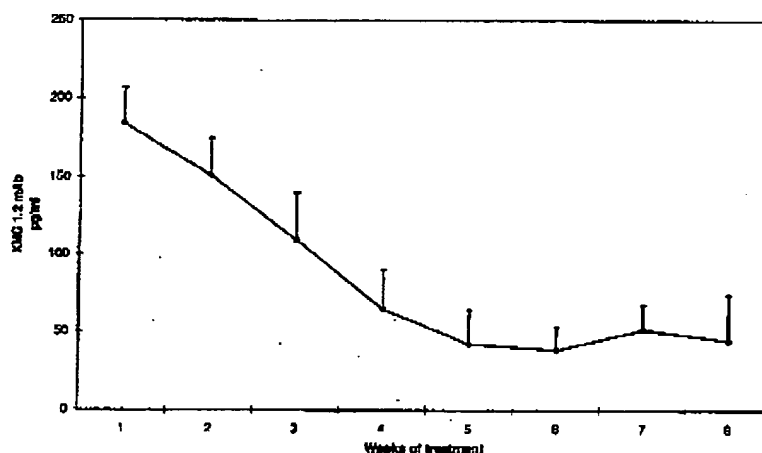


FIG. 4. Formation of murine IgG anti-XMG 1.2 and AN-18 mAbs in NOD mice. Sixteen-week-old NOD mice were bled before and weekly after beginning the treatment with PBS (F), XMG 1.2 (H) or AN-18 (I) anti-IFN $\gamma$  mAb, or sIFN $\gamma$ R (J), and the production of antibody directed against either of the two mAbs or sIFN $\gamma$ R was assessed by solid phase ELISA. Data are representative of seven mice per group. One to 100 diluted sera are shown. Each group is compared to the pretreatment value. a,  $P < 0.001$ ; b,  $P < 0.005$ ; c,  $P < 0.003$ ; d,  $P < 0.01$ ; e,  $P < 0.03$ . SDs are within 60%, with a 32% mean value.

FIG. 5. Levels of unbound XMG1.2 mAb in NOD mice. Sixteen-week-old NOD mice were bled weekly after beginning treatment with XMG 1.2 anti-IFN $\gamma$  mAb, and the levels of unbound concentrations of mAb antibody were assessed by ELISA. Data are shown as the mean  $\pm$  SD.



dent studies (4, 5) and presently confirmed. The reason for this is not known. As previously mentioned, the possibility cannot be ruled out that sIFN $\gamma$ R has, in fact, prevented CY-induced diabetes, but the smaller size of the experimental groups vs. the number of PBS-treated control mice may have blunted statistical significance. Further studies are required

to test this hypothesis with larger numbers of mice and possibly higher doses of sIFN $\gamma$ R. Nonetheless, the higher efficacy of the anti-IFN $\gamma$  mAbs over the sIFN $\gamma$ R in CY-induced diabetes could be due to the peculiar pathophysiology of diabetes in this model and the biological and pharmacokinetic properties of the different specific IFN $\gamma$



inhibitors used. Thus, the exuberant production of endogenous IFN $\gamma$  known to be triggered by CY challenge (4) may have been sufficiently large to supersede the neutralizing capacity of sIFN $\gamma$ R, which may, in turn, have been minimized by its short persistence in the blood. On the other hand, these experimental conditions may have optimized the action of anti-IFN $\gamma$  mAbs for both their long half-life (10–15 days) and the short frame time considered (15 days), within which, as inferred from the data obtained from the spontaneous model, neutralizing antibodies may have only minimally interfered with their action.

Although the development of insulinitis could only be prevented in a clear-cut fashion when specific IFN $\gamma$  inhibitors were administered early in the course of the disease, both sIFN $\gamma$ R and the anti-IFN $\gamma$  mAbs successfully inhibited IDDM development in those NOD mice treated late during the prediabetic period and which exhibited a prevalence and extent of insulinitis comparable to that of control mice. Although a discrepancy between histological and clinical processes was previously observed in NOD mice with other immunotherapeutic approaches (20, 33), this finding underscores the essential role of IFN $\gamma$  in both the afferent and efferent phases of IDDM and shows that the entire diabetogenic process may be prevented if the cytokine is blocked early during disease development. However, the finding that specific IFN $\gamma$  inhibitors can prevent IDDM development even when they are first administered at a late stage of the disease and in the presence of active insulinitis can be important for the clinical setting, where prophylactic interventions can only be performed in those subjects exhibiting metabolic and immunological signs presumably associated with actively ongoing disease.

That anti-IFN $\gamma$  mAbs were ineffective on the insulinitis process in the CY model of diabetes contrasts with other studies in which the anti-IFN $\gamma$  mAb (RA-642) drastically reduces the extent of insulinitis in CY-treated NOD/Whei mice (4) and also prevents insulinitis development in the adoptive transfer model of diabetes in NOD mice (5). Although the different experimental conditions used in this latter model make it difficult to compare the data, the histological discrepancy with the study by Campbell *et al.* (4) may depend on the different colonies of NOD mice, because at 100–120 days of age, the NOD/Lt mice we used exhibit a more severe insulinitis than the NOD/Whei mice used by Campbell *et al.* (34). Thus, the capacity of anti-IFN $\gamma$  mAb to successfully reduce insulinitis in CY-challenged NOD/Whei mice may have been favored by the less advanced, and possibly reversible, phase of  $\beta$ -cell destruction occurring in this colony of NOD mice compared to the Lt counterpart. On the other hand, the inability of IFN $\gamma$  inhibitors to reduce the development of insulinitis when administered under a late prophylactic regimen accords with a study from Jacob *et al.*, in which the DB-1 anti-IFN $\gamma$  mAb administered for 8 consecutive weeks to 7- to 8-week-old female NOD mice did not affect the progression of insulinitis in these mice (35). Taken together, these results show variable effects of IFN $\gamma$  inhibitors on the insulinitic process in the NOD mouse, which may depend on the NOD colony considered, the potency and immunogenicity of the IFN $\gamma$  inhibitor, and the ages of the mice when the treatment is started.

The recurrence of diabetes after treatment withdrawal and

the inability of spleen cells from either anti-IFN $\gamma$ - or sIFN $\gamma$ R-treated animals to protect syngeneic recipients from IDDM development both indicate that blockage of endogenous IFN $\gamma$  neither eliminated autoreactive effectors nor induced suppressor cells. In contrast, in *ex vivo* conditions, SLC from the NOD mice that received either anti-IFN $\gamma$  mAb or, in particular, sIFN $\gamma$ R produced less IL-2 and more IL-6 than SLC from control mice, suggesting a modulatory action of IFN $\gamma$  inhibitors on the cytokine network. This action may have contributed to the beneficial effects, because IL-2 is pathogenically involved in IDDM in NOD mice (36). The possible significance of the up-regulated production of IL-6 is less clear, as this cytokine itself has been incriminated as a possible mediator of  $\beta$ -cell destruction (2, 4). However, IL-6 has been shown to exert powerful antiinflammatory properties both *in vitro* (37) and *in vivo* (38), and these latter effects could have prevailed under the experimental conditions considered. Interestingly, if the modulation of IL-2 and IL-6 production contributed to the antidiabetogenic action of the IFN $\gamma$  inhibitors, the greater immunomodulatory effect of sIFN $\gamma$ R over XMG 1.2 mAb would then accord with the better histological protection offered by the sIFN $\gamma$ R. Although the reason for the greater efficiency of sIFN $\gamma$ R over XMG 1.2 mAb is not known, the progressive reduction of the unbound levels of XMG 1.2 observed in NOD mice upon prolonged treatment anticipates that this effect could depend on a better neutralization of endogenous IFN $\gamma$  by sIFN $\gamma$ R during the last periods of the *in vivo* study.

The possibility of preventing spontaneous IDDM in NOD mice with sIFN $\gamma$ R may be important in the clinical setting, as the complementary DNA encoding the extracellular domain of the receptor was engineered and expressed in both *Escherichia coli* and the insect/baculovirus cell system as a soluble form of the receptor (39). At present, if these data may somehow mirror the impact of sIFN $\gamma$ R therapy in human IDDM, some points can be anticipated to warrant particular attention, including the above-mentioned reversibility of the antidiabetogenic action and the short half-life of this molecule. Certainly, the continuous and possibly life-long treatment with specific IFN $\gamma$  inhibitors may not be clinically feasible, and therapeutic approaches based on short courses of treatment with multiple agents, including other proinflammatory cytokine inhibitors (sTNF-R, sIL-1R, and IL-1 receptor antagonist), antiinflammatory cytokines (IL-10, IL-13, and TGF $\beta$ ), or subtherapeutic doses of cyclosporin A should be considered. Moreover, we have previously underlined how the disadvantage of sIFN $\gamma$ R over anti-IFN $\gamma$  mAb therapy is the shorter half-life of the former. In the murine system we have overcome this problem by generating one mIFN $\gamma$ R-Ig fusion protein that is not immunogenic, possesses a 40 times longer half-life than sIFN $\gamma$ R, and is biologically active *in vivo* in the model of low dose streptozocin-induced diabetes (40). Along this line of reasoning, similar approaches could be used to generate human IFN $\gamma$ R-Ig fusion proteins.

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